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Insulin-Like Growth Factor-I Induces Oocyte Maturational Competence but Not Meiotic Resumption in White Bass (*Morone chrysops*) Follicles In Vitro: Evidence for Rapid Evolution of Insulin-Like Growth Factor Action[1]

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ABSTRACT

A combination of recombinant human (rh) insulin-like growth factor-I (IGF-I) (25 nM) and the maturation-inducing hormone (MIH), 17,20β,21-trihydroxy-4-pregnen-3-one (20β-S; 72.5 nM), induced germinal vesicle breakdown (GVBD) in ovarian follicles of white bass incubated in vitro, whereas a four times greater concentration of each hormone was ineffective alone. These results indicate that IGF-I induces oocyte maturational competence (OMC) but not meiotic resumption in white bass. Culture medium concentrations of 20β-S remained below detection limits for ovarian fragments incubated with rhIGF-I. Actinomycin D blocked GVBD in response to hCG but not to rhIGF-I plus 20β-S, suggesting that IGF-I requires de novo translation but not transcription to induce OMC. Gap junction uncouplers, 1-octanol and 1-heptanol, and the phosphatidylinositiol 3-kinase (PI 3-K) inhibitors, wortmannin and LY 294002, attenuated hCG-, 20β-S-, and rhIGF-I plus 20β-S-induced GVBD. Although these inhibitors reduced hCG-induced progestin release, PI 3-K inhibitors did not alter MIH synthesis in some incubations and addition of 20β-S to the incubations did not fully overcome the effects of either class of inhibitors, suggesting that decreasing MIH production is not their only inhibitory effect on gonadotropin (GtH) action. Our data suggest that gap junctions and PI 3-K activity are necessary for GtH and IGF-I to induce and maintain OMC in white bass. The induction of OMC but not meiotic resumption by IGF-I in white bass, compared with the induction of meiotic resumption but not OMC by IGF-I discovered in the congeneric striped bass suggests rapid evolution of the reproductive actions of IGF-I among temperate basses (genus *Morone*).

follicle, growth factors, mechanisms of hormone action, meiosis, signal transduction

INTRODUCTION

In teleost fishes and amphibians, ovarian follicle maturation consists of two stages [see reviews 1, 2]. The first stage is the acquisition of oocyte maturational competence (OMC), in which the follicle acquires the ability to produce and respond to the maturation-inducing steroid hormone (MIH). The second stage is meiotic resumption, the release of the oocyte from meiotic arrest in response to MIH produced by the somatic follicle cells. The MIH induces meiotic resumption by activating a ubiquitous maturation promoting factor (MPF) [3, 4]. Although ovarian follicle maturation is generally considered to be under the control of LH, and in turn, MIH, evidence suggests that insulin-like growth factors (IGFs) also are involved. An ovarian IGF system is present in maturing ovarian follicles of teleost fishes. IGF-I was detected by immunolocalization in the ovary of red seabream (*Pagrus major*), although IGF-I immunoreactivity was lower during follicle maturation than during early stages of vitellogenic oocyte growth [5]. Expression of mRNA for IGF-I, IGF-II, IGF-I-receptor, and IGF-binding protein-2 was detected in maturing and preovulatory follicles of fish [6–10]. In rainbow trout (*Oncorhynchus mykiss*), both IGF-I and IGF-II gene expression increased as maturation progressed, whereas there were no statistically significant changes in IGF-I-receptor or IGF-binding protein-2 gene expression [9, 10].

Most of what is known about IGF actions during ovarian follicle maturation in teleost fishes comes from in vitro investigations, and the results of these studies suggest that there are species differences in IGF-I actions. IGFs can induce OMC and meiotic resumption in some species, including the red seabream, or meiotic resumption via a steroid independent pathway, as in the mummichog (*Fundulus heteroclitus*) and in the striped bass (*Morone saxatilis*) [11–16]. The identity of follicular changes underlying acquisition of OMC remains speculative, although evidence strongly suggests that increases in membrane MIH receptors and gap junctional contacts between the follicle cells and the oocyte (heterologous) and among the granulosa cells (homologous) are involved [1, 17–20]. Increases in the number of receptors for the MIH and increases in gap junction contacts appear to be components of OMC induced by IGF-I in fishes [14]. Shifts in steroid synthesis toward MIH production also can be induced by IGFs in salmonids, red seabream, and striped bass [12, 15, 21, 22]. IGFs have not previously been shown to induce OMC without meiotic resumption in any fish species.

Although actions of IGFs on follicle maturation have been studied in few but diverse species of vertebrates and differences in IGF-I actions among species have been noted, little work has been conducted to address the bases of these species differences. Variation in IGF actions among vertebrates may derive from phylogenetic distance or regulatory requirements of divergent reproductive strategies. The present article describes a series of experiments con-

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ducted to characterize the effects of IGF-I on OMC, meiotic resumption, and follicular steroidogenesis in white bass (*Morone chrysops*), for comparison to the maturational actions of IGF-I previously described for the congeneric striped bass [15, 16, 23]. The white bass is most closely related to the striped bass among the several species in this very young genus of temperate basses [24]. White bass are group-synchronous multiple-clutch spawners with demersal eggs, whereas striped bass are group-synchronous single-clutch spawners with pelagic eggs. Effects of gonadotropin (GtH) and the MIH on OMC and meiotic resumption in white bass were examined for comparison to the actions of IGF-I in white bass and striped bass. The reproductive physiology of both striped bass and white bass have been investigated in detail, as they are important fisheries and aquaculture species, and the hybrid of these species is an important cultivar known as the hybrid striped bass. The regulation of follicle maturation by GtH and the MIH has been described previously for both species [see review, 25] and, as noted, regulation of follicle maturation by IGFs has been investigated in striped bass.

In the present study, we examined the maturational effects of recombinant human IGF-I (rhIGF-I), a gonadotropin (hCG), and the MIH in *Morone*, 17,20β,21-trihydroxy-4-pregnen-3-one (20β-S) [26–29] on germinal vesicle breakdown (GVBD) using in vitro incubations of ovarian fragments. The effects of the peptides in combination with MIH also were evaluated. The dissolution of the oocyte germinal vesicle (GVBD) is commonly used as an indicator of the resumption of meiosis. The actions of a gonadotropin and of the MIH were evaluated for comparison to IGF-I actions. The effects of inhibitors of transcription, translation, and activity of phosphatidyinositol 3-kinase (PI 3-K), a key enzyme in the IGF-I signal transduction pathway, and of gap junction uncouplers on the maturational actions of the various hormones also were examined to address mechanisms of action of IGF-I in ovarian follicle maturation, as described previously for the striped bass [15, 16].

MATERIALS AND METHODS

Chemicals

The rhIGF-I was purchased from GroPep, Inc. (Adelaide, Australia). The steroids, 20β-S and 17,20β,dihydroxy-4-pregnen-3-one (17,20β-P), were purchased from Steraloids, Inc. (Wilton, NH). Bovine insulin, hCG, actinomycin D, cycloheximide, 1-heptanol, 1-octanol, ethanol, and wortmannin used in the in vitro cultures and the estradiol-17β (E_2) and testosterone (T) used as standards for radioimmunoassays (RIA) were purchased from Sigma Chemical Co. (St Louis, MO). The LY 294002 was purchased from Calbiochem Corp. (La Jolla, CA). The rhIGF-I and bovine insulin were dissolved in 10 mM HCl and the steroids were dissolved in absolute ethanol at 1 mg/ml. The wortmannin and LY 294002 were dissolved in dimethyl sulfoxide to make 1 mM and 50 mM stocks, respectively. Actinomycin D, cycloheximide, 1-heptanol, and 1-octanol were dissolved directly in culture medium. Solvents for these chemicals never exceeded 0.1% (v/v) of the volume of the culture medium.

Animals

Most of the adult white bass that served as ovarian donors were obtained from domestic broodstocks maintained at the Pamlico Aquaculture Field Laboratory (PAFL), North Carolina State University. Tissues from captive fish maintained at Carolina Fisheries (CF), Aurora, NC, were used for a single study presented in this article; a comparison of wortmannin and LY 294002 effects on hCG- and 20β-S-induced GVBD in MIH-competent ovaries. The white bass from PAFL were 2–3 yr old, F2 generation, domesticated broodstock, and the fish from CF were caught in previous seasons from the Ohio River and reconditioned for spawning at CF. The fish were brought into the hatcheries at both locations in April and were thereafter maintained at 10–12°C to prevent final maturation or atresia, a procedure commonly referred to as coldbanking [25]. The PAFL fish were moved in early April from a brackish water pond (~10 ppt) supplied with water from a nearby creek into indoor tanks. The indoor tanks were fed with water from the same source as the pond and were equipped with biofilters, sand filters, and chillers. The water temperature in the pond and tanks was 20°C on the day the fish were transferred. The water temperature in the tanks was then lowered 1–2°C per day until it reached 12°C. Photoperiod remained at ambient conditions. The fish were fed a 38% protein commercial hybrid striped bass feed (Southern States, VA) every 2–3 days. Fish were treated similarly at CF, where they were maintained under ambient photoperiod in a recirculating system filled with well water and fitted with water chillers. To ensure water quality, the recirculating water systems were supplied with inflow of new water sufficient to replace the entire system volume once each few days. White bass were deeply anesthetized before handling [28], and all experiments involving live fish were carried out in accordance with the *1996 Guide for Care and Use of Laboratory Animals* published by the National Research Council.

In Vitro Bioassay

In vitro incubations of ovarian follicles and subsequent evaluation of oocyte maturation stages followed our routine procedures [26, 28]. Females with fully grown ovaries were warmed from ~10°C to ~20°C over a 12- to 24-h period, and they were then allowed to remain at the warmer temperature for 1–5 days before their ovaries were removed for incubation. Depending on the specific experiment, fish either were not injected with hormone or they were primed with an intramuscular injection of hCG (100 IU/kg body weight) to induce OMC [27, 28] before the removal of their ovaries to obtain tissue for culture. Unprimed females with maximum ovarian follicle diameters greater than 600 μm but displaying no signs of lipid droplet coalescence in their ooplasm were selected as ovarian donors for studies requiring early-stage follicles (stage 1) [19] that have not acquired OMC and are unresponsive to MIH in vitro, or were MIH incompetent [28]. Fish with oocytes having a centrally located germinal vesicle and just initiating lipid droplet coalescence in their ooplasm (stage 2) [19] were selected when more mature tissues that had acquired OMC and were responsive to MIH in vitro, or were MIH competent [28], were desired for culture. Although it was usually necessary to prime fish with hCG to obtain ovaries that were mature enough to respond to MIH in vitro, some fish used in our studies reached this stage of maturity without receiving a hormone injection.

The ovaries were excised, placed into cold (4°C) media consisting of Cortland balanced physiological saline buffered with 15 mM HEPES, adjusted to pH 7.5, and supplemented with 1 g D-glucose/L, 0.1% BSA, 100 000 U sodium penicillin G/L, and 100 mg streptomycin S/L. The ovaries were then fragmented with scissors and fragments weighing approximately 0.1 g were placed into individual wells of a Falcon 24-well culture plate (Becton Dickinson, Franklin Lakes, NJ) containing 1 ml of control medium. After 1 h of preincubation, the control medium was exchanged for the same volume of control medium, or medium containing inhibitors (cycloheximide, actinomycin D, 1-heptanol, 1-octanol, wortmannin, or LY 294002), and the tissues were given an additional 2 h of preincubation. For each experiment, the number of hours of incubation following preincubations is presented in the *Results*. For incubation, tissues were moved to new plates containing control medium or experimental medium containing hormones, inhibitors, or both. Cultures were placed in a Dubnoff shaking incubator at 22°C under air. Upon termination of the experiment, the medium was aspirated, centrifuged, and stored at −80°C for later measurement of steroid hormone concentrations by RIA. The follicles were then placed in an oocyte-clearing solution of ethanol:formalin:acetic acid (6:3:1 v/v) [30] (chemicals purchased from Fisher Scientific) and examined under a stereomicroscope to detect and enumerate the incidence of GVBD. If the oocyte cytoplasm was too dark to reliably see the nucleus, a drop of glycerol was placed onto the oocytes to further clear their cytoplasm.

Hormone Measurement

Steroid hormones in culture medium were measured in duplicate using highly specific RIAs for 20β-S, 17,20β-P, E_2, and T as described previously and validated for use with the culture medium [26, 27, 31]. Aliquots of culture medium were triple extracted with ethyl ether, dried under a stream of nitrogen gas at 37°C, and resuspended in RIA buffer for assay. 20β-S, 17,20β-P, and E_2 were each extracted from 100 μl of culture medium per replicate, and T was extracted from 30 μl of culture medium. Hormone detection limits of the assays, defined as the minimum quantity

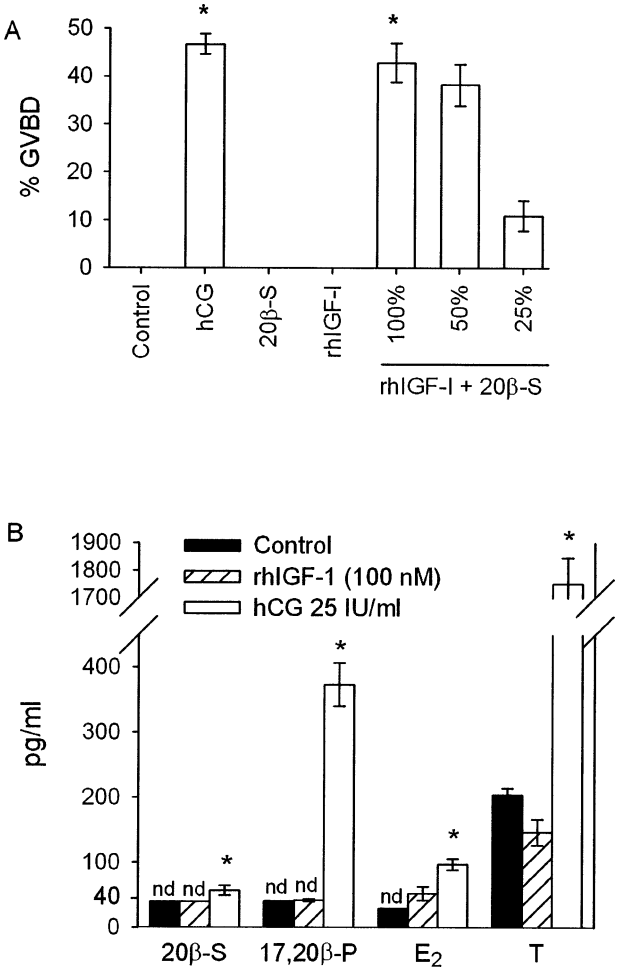

FIG. 1. Percentage GVBD in MIH-incompetent ovarian fragments exposed for 12 h to medium without added hormone (control), 290 nM 20β-S, 25 IU hCG/ml, 100 nM rhIGF-I, or decreasing percentages of a combination 100 nM IGF-I plus 290 nM 20β-S (**A**). Steroid concentrations measured in the medium of treatment groups indicated (**B**). Each bar represents the mean of triplicate incubations. Vertical brackets indicate SEM. Asterisks denote values significantly different from those shown for tissues incubated without exogenous hormone ($P \leq 0.05$). Values for samples below the detection limits of the steroid assays were calculated as that detection limit. nd, Nondetectable.

that could be statistically distinguished from bound zero values, were 40 pg/ml for 20β-S and 17,20β-P, 30 pg/ml for E_2, and 100 pg/ml for T.

Data Analysis

Data were obtained from a number of experiments using several fish from different sources. The data presented in each figure were collected from a single animal and each data point represents the mean of three replicate incubations unless otherwise noted. Only results from experiments that were successfully repeated using tissues from a second animal are shown. Percentage data were arcsine transformed to normalize data distributions before statistical analysis. Two-way ANOVA was used to test for differences among treatments in the percentage of oocytes completing GVBD and in medium steroid concentrations. ANOVA was followed by the Student-Newman-Keuls test [32] using the statistical software SigmaStat (SYSTAT Software Inc., Richmond, CA). The Mann-Whitney test was used when comparing only two means in cases where one of the means had two or more zero values [32]. For all analyses, the a priori level of statistical significance used was $P \leq 0.05$.

RESULTS

Effects of rhIGF-I and 20β-S Interactions on Follicle Maturation

During 60 h of incubation, oocytes in MIH-competent ovarian follicles that were cultured without hormone or with 1, 10, 100 or 1000 nM rhIGF-I or bovine insulin failed to undergo GVBD. In contrast with these results, over 50% of oocytes from the same ovaries that were exposed to 290 nM 20β-S completed GVBD within 15 h of incubation (data not shown). These observations support that IGF-I cannot induce GVBD via a steroid-independent pathway in white bass. The effects of incubating MIH-incompetent ovarian fragments with hCG, rhIGF-I, or 20β-S alone were compared with those obtained by incubating tissues with combinations of rhIGF-I plus 20β-S (Fig. 1A). Ovarian follicles completed oocyte GVBD during incubation with 25 IU hCG/ml but not with 100 nM rhIGF-I or 290 nM 20β-S. Oocytes in ovarian fragments incubated with a combination of rhIGF-I plus 20β-S equal to 25% of the concentrations of the hormones that failed to induce GVBD when used alone also underwent GVBD. These data suggest an interaction of rhIGF-I and 20β-S that goes beyond a simple additive effect of hormone dosage. The data are consistent with a scenario in which rhIGF-I induces OMC and 20β-S then activates MPF. Medium concentrations of the MIH (20β-S) and of 17,20β-P, E_2, and T were elevated in response to hCG, whereas the addition of rhIGF-I to the cultures had no effect on medium concentrations of these steroids, with all but T concentrations remaining below the detection limits of the RIAs (Fig. 1B).

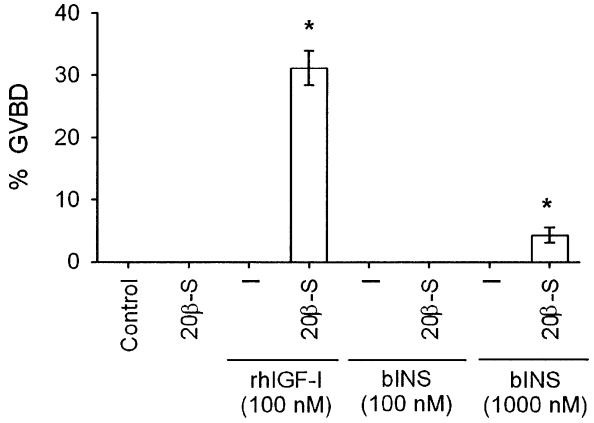

FIG. 2. Percentage GVBD in MIH-incompetent ovarian fragments exposed for 26 h to medium without added hormone (control), 100 nM rhIGF-I, or 100 or 1000 nM bovine insulin (bINS) with or without 290 nM 20β-S added to the culture medium. Each bar represents the mean of triplicate incubations. Vertical brackets indicate SEM. Asterisks denote values significantly different from those shown for tissues incubated without exogenous hormone ($P \leq 0.05$).

Comparison of the Effects of rhIGF-I and Bovine Insulin on Follicle Maturation

Incubation of MIH-incompetent ovarian fragments with 100 nM rhIGF-I plus 290 nM 20β-S or with 1000 nM bovine insulin plus 290 nM 20β-S induced oocyte GVBD, whereas oocytes in follicles incubated with 100 or 1000 nM rhIGF-I or bovine insulin alone or with 100 nM bovine insulin plus 290 nM 20β-S failed to complete GVBD (Fig. 2). The percentage of oocytes that completed GVBD when incubated with 1000 nM bovine insulin plus 290 nM 20β-S was lower than the percentage of oocytes completing GVBD when incubated with 100 nM rhIGF-I plus the same concentration of 20β-S.

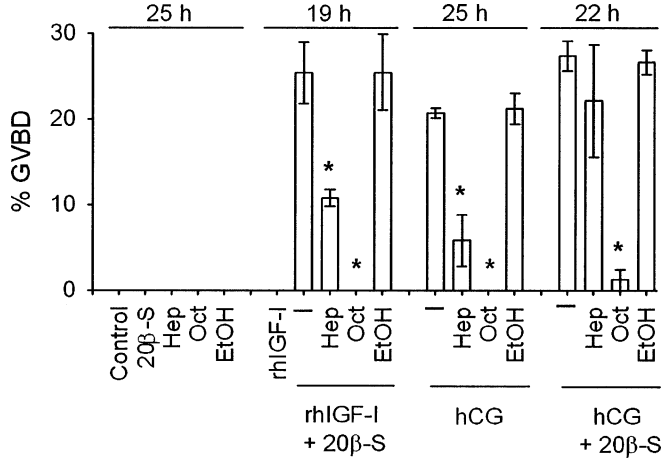

FIG. 3. Percentage GVBD in MIH-incompetent ovarian fragments exposed to medium with various combinations of 1 mM of the *n*-alkanols 1-octanol (Oct) or 1-heptanol (Hep), 2 mM of EtOH, 290 nM 20β-S, 100 nM rhIGF-I, and 25 IU hCG/ml. Tissues were incubated for 19, 22, or 25 h as indicated. Each bar represents the mean of triplicate incubations. Vertical brackets indicate SEM. Asterisks denote values significantly different from those shown for the same hormone treatment without *n*-alkanols or EtOH ($P \leq 0.05$).

Effects of Gap Junction Uncouplers, 1-Heptanol, and 1-Octanol on Hormone-Induced Follicle Maturation

The effects of *n*-alkanols that function as gap-junction uncouplers (1-heptanol and 1-octanol) on GVBD induced by rhIGF-I, hCG, 20β-S, or by combinations of these hormones were investigated using both MIH-incompetent and MIH-competent ovarian fragments. MIH-incompetent tissues were incubated with 100 nM rhIGF-I, rhIGF-I plus 290 nM 20β-S, 25 IU hCG/ml, or hCG plus 20β-S, with or without 1 mM of 1-heptanol, 1-octanol, or 2 mM EtOH, which served as a control for the *n*-octanols (Fig. 3). MIH-competent tissues were incubated with the same concentrations of hCG, hCG plus 20β-S, or 20β-S, with or without 1 mM of the *n*-alkanols or 2 mM ethanol (EtOH) (Fig. 4). In the MIH-incompetent tissues, oocyte GVBD was induced by a combination of IGF-I plus 20β-S, hCG, or hCG plus 20β-S but not by IGF-I or 20β-S alone. In MIH-competent tissues, hCG, 20β-S, and hCG plus 20β-S induced oocyte GVBD at a higher frequency than observed in control cultures, whereas rhIGF-I alone was without effect. In either set of experiments, the actions of all hormones and hormone combinations on oocyte GVBD were inhibited by 1 mM 1-octanol and were unaffected by 2 mM EtOH. Incubation of ovarian fragments with 1 mM 1-heptanol also attenuated induction of GVBD by hCG, rhIGF-I plus 20β-S, and 20β-S, but 1-heptanol did not significantly impair GVBD induced by 20β-S or hCG plus 20β-S in either set of experiments. These findings suggest that coupled or coupling of gap junctions is required for acquisition of OMC in the white bass and that the coupling of gap junctions is induced or maintained by GtH and IGF-I.

Concentrations of MIH 20β-S and its putative precursor, 17,20β-P [29], were measured in the culture medium from both sets of experiments. For all treatments of MIH-incompetent tissues, medium 20β-S concentrations were below detection limits and, although there was a trend toward a reduction in 17,20β-P release in response to 1-octanol, this trend was not statistically significant. Medium 17,20β-P concentrations were 249 and 164 pg/ml for MIH-incompetent tissues incubated with hCG and hCG plus 1-octanol,

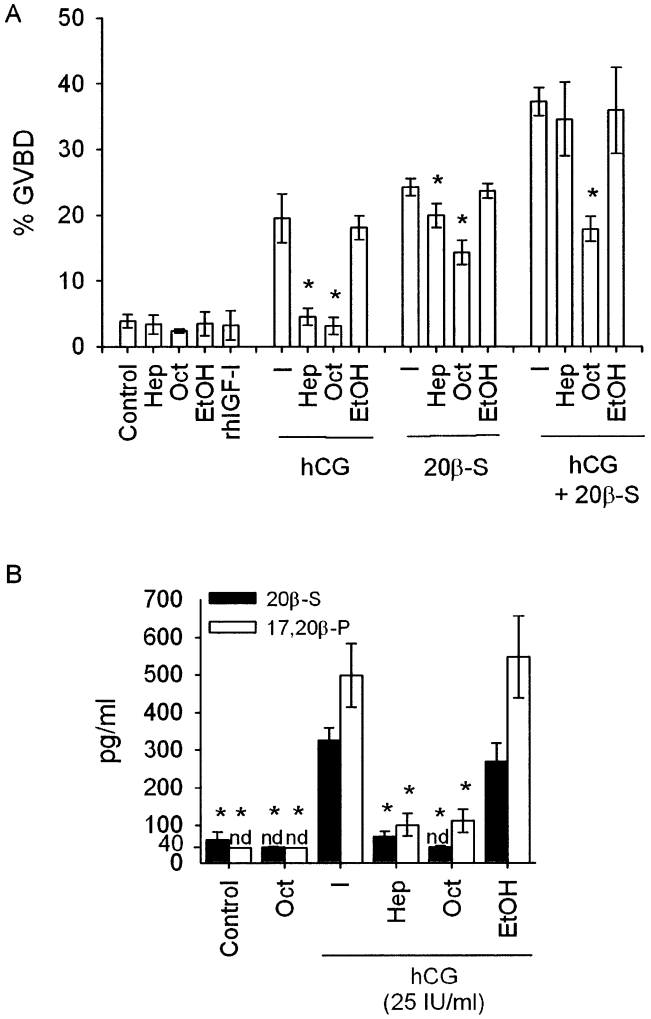

FIG. 4. Percentage GVBD in MIH-competent ovarian fragments exposed for 12 h to medium with various combinations of 1 mM of the *n*-alkanols 1-octanol (Oct) or 1-heptanol (Hep), 2 mM of EtOH, 290 nM 20β-S, 100 nM rhIGF-I, and 25 IU hCG/ml (**A**). Steroid concentrations measured in the medium of treatment groups indicated (**B**). Each bar represents the mean of triplicate incubations. Vertical brackets indicate SEM. Asterisks denote values significantly different from those shown for the same hormone treatment without *n*-alkanols or EtOH ($P \leq 0.05$). Values for samples below the detection limits of the steroid assays were calculated as that detection limit. nd, Nondetectable.

respectively ($P = 0.06$; data not shown). Medium concentrations of both 20β-S and 17,20β-P were elevated in response to hCG in cultures of MIH-competent tissues, and both 1-heptanol and 1-octanol inhibited release of these steroids into the culture medium (Fig. 4B). Although the *n*-alkanols could inhibit MIH production, the observation that the addition of MIH to the culture medium did not fully overcome their inhibition of oocyte GVBD suggests that maintenance of gap junctions is part of IGF-I- and GtH-induced OMC and that functional gap junctions are required for maximal activity of the MIH.

Effects of Cycloheximide and Actinomycin D on Hormone-Induced Follicle Maturation

The effects of the translation inhibitor, cycloheximide, and the transcription inhibitor, actinomycin D, on rhIGF-I plus 20β-S- and hCG-induced oocyte GVBD were examined. Ovarian fragments were incubated for 22 h with 100

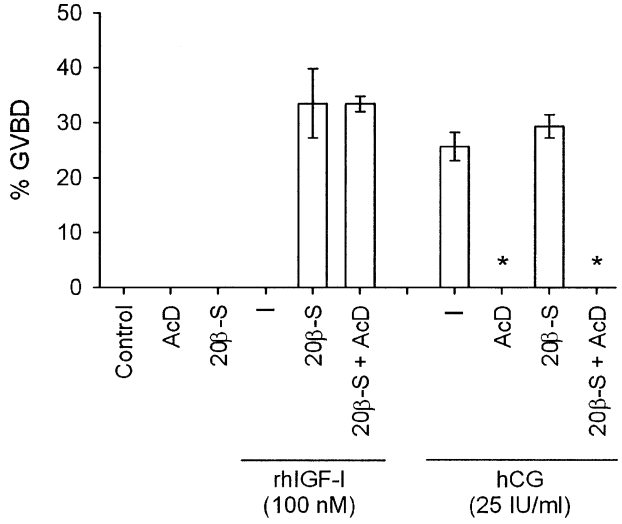

FIG. 5. Percentage GVBD in MIH-incompetent ovarian fragments exposed for 22 h to medium without added hormone (control), 100 nM rhIGF-I or 25 IU hCG/ml alone, or with 290 nM 20β-S, all with or without 1 μg/ml actinomycin D (AcD) added to the culture medium. Each bar represents the mean of triplicate incubations. Vertical brackets indicate SEM. Asterisks denote values significantly different from those shown for the same hormone treatment without actinomycin D ($P \leq 0.05$).

nM rhIGF-I plus 290 nM 20β-S or with 25 IU hCG/ml, with or without 1 μg cycloheximide/ml. Cycloheximide decreased the percentage of oocytes completing GVBD induced by either hCG or the combination of rhIGF-I plus 20β-S from above 40% to 0% (data not shown). Induction of OMC by rhIGF-I or hCG, synthesis of MIH in response to hCG, or activation of MPF by 20β-S may have been inhibited by cycloheximide. Actinomycin D at 1 μg/ml was without effect on 100 nM rhIGF-I plus 290 nM 20β-S-induced GVBD, but it inhibited GVBD induced by 25 IU hCG/ml alone or in combination with 290 nM 20β-S (Fig. 5). These observations suggest that IGF-I can induce OMC

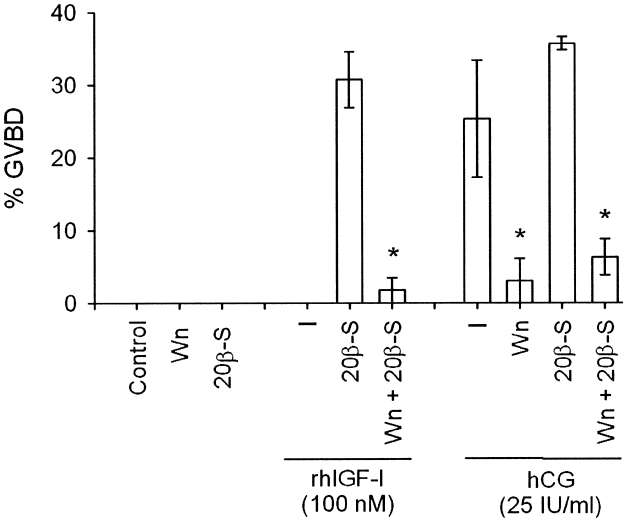

FIG. 6. Percentage GVBD in MIH-incompetent ovarian fragments exposed for 36 h to medium without added hormone (control), 100 nM rhIGF-I or 25 IU hCG/ml alone or with 290 nM 20β-S, all with or without 100 nM wortmannin (Wn) added to the culture medium. Each bar represents the mean of triplicate incubations. Vertical brackets indicate SEM. Asterisks denote values significantly different from those shown for the same hormone treatment without wortmannin ($P \leq 0.05$).

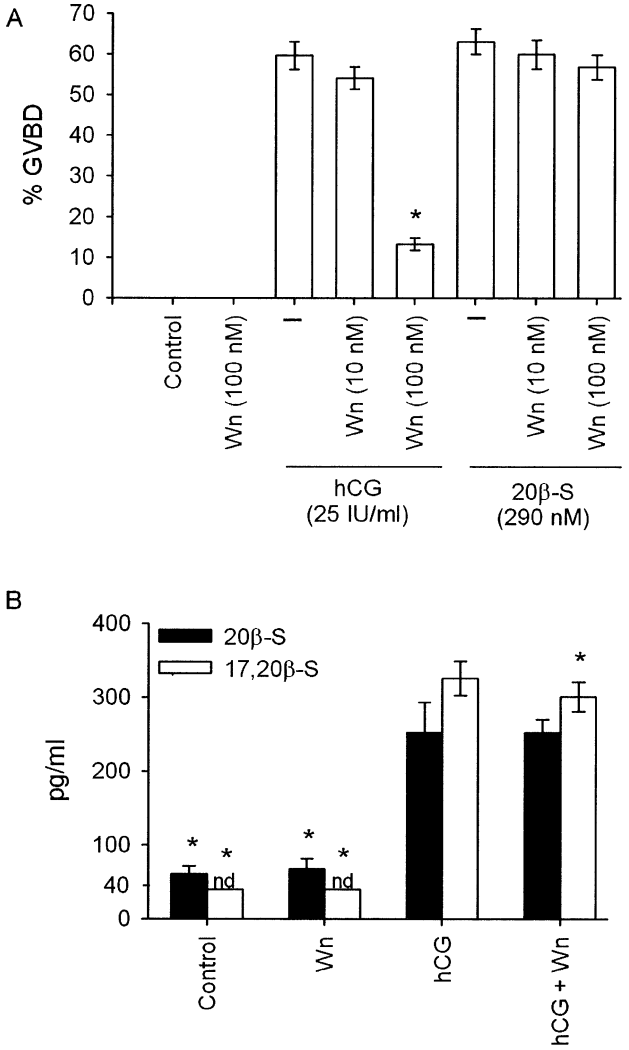

FIG. 7. Percentage GVBD in MIH-competent ovarian fragments exposed for 18 h to medium without added hormone (control), 25 IU hCG/ml or 290 nM 20β-S with or without 10 or 100 nM wortmannin (Wn) added to the culture medium (**A**). Steroid concentrations measured in the medium of treatment groups indicated (**B**). Each bar represents the mean of triplicate incubations. Vertical brackets indicate SEM. Asterisks denote values significantly different from those shown for the same hormone treatment without wortmannin ($P \leq 0.05$). Values for samples below the detection limits of the steroid assays were calculated as that detection limit. nd, Nondetectable. There was a significant difference between concentrations of 17,20β-P for tissues incubated with hCG compared with tissues incubated with hCG plus 100 nM wortmannin detected by two-way ANOVA, although the data shown are from the replicate with the smaller difference in the means.

without de novo synthesis of RNA, whereas gene transcription is required for GtH to induce OMC.

Effects of the PI 3-K Inhibitor, Wortmannin, on Hormone-Induced Follicle Maturation

The effects of the PI 3-K inhibitor, wortmannin, on induction of oocyte GVBD in MIH-incompetent ovarian fragments by rhIGF-I plus 20β-S, hCG, and hCG plus 20β-S were examined (Fig. 6). The effects of wortmannin on induction of GVBD by hCG and 20β-S also were examined using MIH-competent tissues (Fig. 7A). In both sets of experiments, 20β-S and 17,20β-P were measured in the culture medium of tissues incubated without exogenous 20β-S (Fig. 7B). MIH-incompetent ovarian fragments were in-

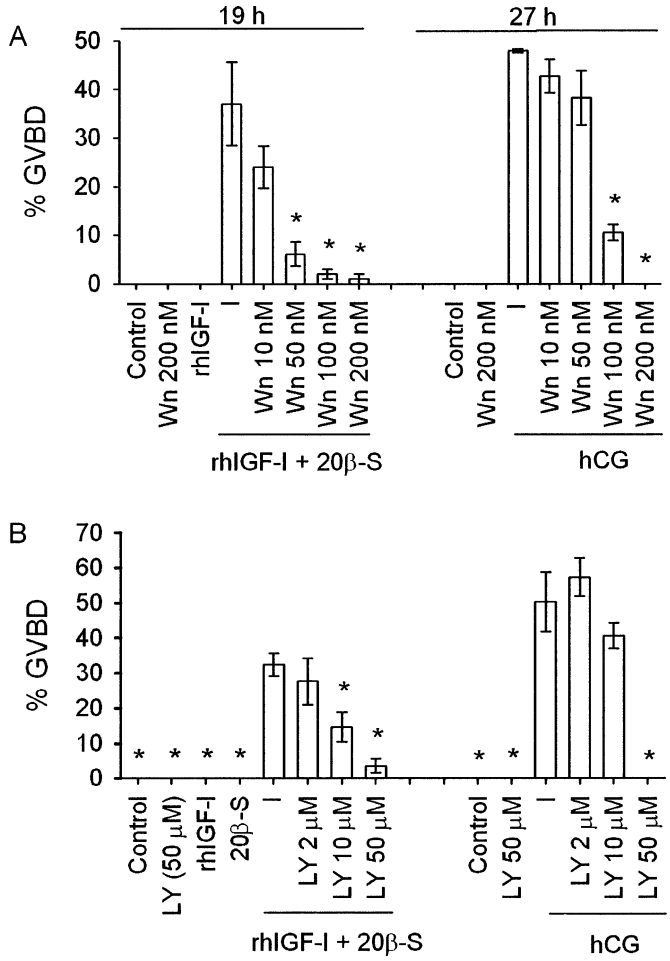

FIG. 8. Percentage GVBD in MIH-incompetent ovarian fragments exposed to medium without added hormone (control), 100 nM rhIGF-I, 100 nM rhIGF-I plus 290 nM 20β-S, or 25 IU hCG/ml alone or with or without graded concentrations of wortmannin (Wn) (**A**) or LY 294002 (LY) (**B**) added to the culture medium. Tissues were incubated for 19 or 27 h as indicated. Each bar represents the mean of triplicate incubations. Vertical brackets indicate SEM. Asterisks denote values significantly different from those shown for the same hormone treatment without inhibitor ($P \leq 0.05$).

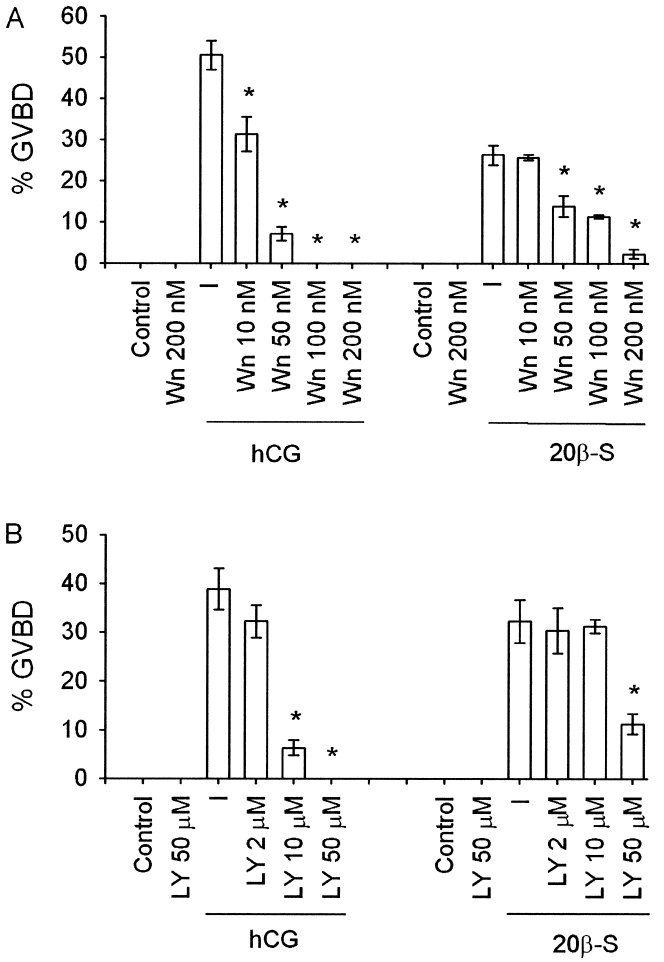

FIG. 9. Percentage GVBD in MIH-competent ovarian fragments exposed for 27 h to medium without added hormone (control), 100 nM rhIGF-I, 100 nM rhIGF-I plus 290 nM 20β-S, or 25 IU hCG/ml alone or with or without graded concentrations of wortmannin (Wn) (**A**) or LY 294002 (LY) (**B**) added to the culture medium. Each bar represents the mean of triplicate incubations. Vertical brackets indicate SEM. Asterisks denote values significantly different from those shown for the same hormone treatment without inhibitor ($P \leq 0.05$).

cubated with 25 IU hCG/ml, 100 nM rhIGF-I, 290 nM 20β-S, and with the peptides in combination with 20β-S, with or without 100 nM wortmannin. Wortmannin inhibited induction of oocyte GVBD by rhIGF-I plus 20β-S, hCG, and hCG plus 20β-S (Fig. 6). Incubation of MIH-incompetent follicles with 100 nM rhIGF-I alone did not induce oocyte GVBD. These findings suggest that PI 3-K activity is required for induction of OMC by both IGF-I and GtH. The addition of 20β-S to the culture medium along with rhIGF-I or hCG did not overcome the inhibition of GVBD by wortmannin, suggesting that the inhibition of GVBD that ensues from blocking PI 3-K activity is not due solely to attenuated MIH production. Concentrations of 20β-S and 17,20β-P were nondetectable in culture medium of MIH-incompetent tissues incubated with rhIGF-I or without exogenous hormone. Incubation of MIH-incompetent tissue with hCG increased medium 17,20β-P concentrations to 242 pg/ml, and addition of wortmannin to these cultures reduced 17,20β-P concentrations to 187 pg/ml (data not shown; $P \leq 0.05$). Because concentrations of 20β-S in these cultures were usually below RIA detection limits, similar statistical comparisons could not be made with regard to medium 20β-S concentrations.

Induction of oocyte GVBD by hCG in MIH-competent ovarian fragments was inhibited by 100 nM wortmannin but not by 10 nM wortmannin, supporting the concept that PI 3-K activity is important for maintenance of OMC by GtH. A trend toward attenuation of 20β-S-induced GVBD by wortmannin was not significant ($P = 0.43$) for the concentrations of wortmannin that were tested (Fig. 7A). Incubation of MIH-competent tissues with hCG increased levels of 20β-S, 17,20β-P, E_2, and T in the culture medium. Wortmannin had no effect on the elevation of 20β-S levels by hCG, but 17,20β-P levels were significantly reduced by wortmannin in these same cultures (Fig. 7B). Thus, a reduction in MIH was not required for wortmannin to inhibit hCG-induced GVBD. Wortmannin at 100 nM also attenuated hCG-induced E_2 production by MIH-competent tissues, reducing medium E_2 levels from 200 to 158 pg/ml, whereas T levels increased from 7,509 to 12 706 pg/ml with the addition of wortmannin to these cultures (data for E_2 and T not shown; $P \leq 0.05$).

Comparison of the Effects of the PI 3-K Inhibitors, Wortmannin, and LY 294002, on Hormone-Induced Follicle Maturation

The effects of an additional PI 3-K inhibitor, LY 294002, and a wider range of wortmannin concentrations on hCG- and rhIGF-I plus 20β-S-induced oocyte GVBD in MIH-incompetent tissues (Fig. 8, A and B) and on hCG- and 20β-S-induced GVBD in MIH-competent tissues were examined (Fig. 9, A and B). Tissues were incubated with combinations of 100 nM rhIGF-I, 25 IU hCG/ml, and 290 nM 20β-S. In MIH-incompetent tissues, induction of oocyte GVBD by hCG or rhIGF-I plus 20β-S was inhibited by 50 nM wortmannin but not by 10 nM wortmannin (Fig. 8A) and by 10 μM LY 294002 but not by 2 μM LY 294002 (Fig. 8B). These data are consistent with PI 3-K activity being required for induction of OMC. In the MIH-competent tissues, hCG-induced GVBD was inhibited by 10 nM wortmannin whereas 50 nM was the lowest concentration of wortmannin to inhibit 20β-S action (Fig. 9A). Similarly, in MIH-competent tissues, hCG-induced GVBD was inhibited by 10 μM LY 294002, whereas 50 μM LY 294002 was required to significantly attenuate 20β-S-induced GVBD (Fig. 9B). These results are consistent with the concept that PI 3-K activity is required for maintenance of OMC.

In each of the studies, 20β-S and 17,20β-P were measured in the culture medium of tissues incubated without hormones or inhibitors or of tissues incubated in the presence of rhIGF-I, hCG, or hCG plus 50 μm LY 294002. For all treatments, medium concentrations of 20β-S were below RIA detection limits and concentrations of 17,20β-P were below detection limits for all treatments except those involving hCG. Culture medium levels of 17,20β-P were increased by hCG from below 40 pg/ml to 503 and 396 pg/ml for MIH-incompetent and MIH-competent tissues, respectively, and were reduced by addition of LY 294002 to 203 and 127 pg/ml for MIH-incompetent and MIH-competent tissues, respectively (data not shown; $P \leq 0.05$).

DISCUSSION

The results of the present study show that IGF-I can induce OMC in white bass ovarian follicles, but it cannot induce resumption of meiosis in oocytes independently of the MIH. These IGF-I actions in white bass stand in sharp contrast with IGF-I actions on ovarian follicle maturation in the congeneric striped bass. We previously discovered that IGF-I induces resumption of meiosis of striped bass oocytes, but it cannot induce OMC in this species [15, 16, 23]. Thus, the actions of IGF-I leading to follicle maturation differ between these closely related *Morone* species, indicating that mechanisms for regulation of follicle maturation by IGF-I can evolve rapidly. Unlike the case in striped bass, IGF-I treatment did not induce detectable changes in steroid production by white bass ovarian follicles. In striped bass, IGF-I increased 20β-S and E_2 concentrations and decreased T concentrations measured in the culture medium of ovarian tissue incubated in vitro [15]. Similar, albeit nonsignificant, trends in E_2 and T measured in the medium in response to IGF-I were noticeable in our white bass studies. However, culture medium concentrations of 20β-S always were below RIA detection limits when white bass ovarian tissues were incubated with IGF-I. Therefore, a clear difference between the two *Morone* species in the effects of IGF-I on follicular steroidogenesis is not certain. The disparate maturational actions of IGF-I in the two species appear to share some common mechanisms in that they require PI 3-K activity and de novo protein synthesis but not gene transcription. In addition, the induction of OMC by IGF-I in white bass requires functional gap junctions, which is also the case for IGF-I- or GtH-induced OMC in other species, including the striped bass [1, 15]. There were no apparent differences in MIH or GtH actions on follicle maturation between the two species of temperate basses.

In the first part of this study, we characterized actions of IGF-I leading to follicle maturation in white bass ovarian fragments. In MIH-incompetent tissues, rhIGF-I or 20β-S alone were without effect on oocyte GVBD. However, a combination of the two hormones at concentrations much lower than those that failed to induce GVBD independently was capable of inducing GVBD in oocytes from MIH-incompetent tissues (Fig. 1). These data suggest an interaction between the actions of IGF-I and the MIH that goes beyond a simple dose-related additive effect of the hormones; their combined effect supports the concept that IGF-I stimulates OMC and 20β-S, then induces GVBD. Combined actions of IGF-I plus MIH leading to greater effects than either hormone administered alone and sometimes occurring even when MIH alone was impotent for inducing GVBD led Kagawa and colleagues to draw the same conclusion for the red seabream—that IGF-I induces OMC [11, 12]. In striped bass, IGF-I-induced resumption of meiosis was not as robust as GtH-induced oocyte GVBD when tissues were fully responsive to GtH, suggesting that IGF-I was not able to induce all of the components of follicle maturation stimulated by GtH and the MIH [15, 16, 23]. In the present study, the percentage of follicles completing oocyte GVBD in cultures containing a combination of rhIGF-I plus 20β-S was similar to that occurring in cultures containing hCG plus 20β-S, over a similar incubation period (Figs. 3, 5, and 6), suggesting that IGF-I can fully induce OMC in white bass.

Unlike the case in red seabream, where IGF-I also can induce both OMC and oocyte GVBD without added MIH, we did not observe GVBD occurring in response to IGF-I alone in any of our incubations, even for MIH-competent tissues after 60 h of incubation with 100 nM rhIGF-I (data not shown), or when there was some spontaneous GVBD in the control tissues (Fig. 4). To the best of our knowledge, this clear separation of IGF-I actions in ovarian follicle maturation in white bass, the capability to induce OMC and the inability to induce GVBD, has not been observed in any other vertebrate species. This separation makes the white bass a potentially exceptional model with which to investigate the physiological mechanisms for acquisition of OMC by maturing ovarian follicles.

In the present study, IGF-I alone did not appear to strongly alter steroid production by white bass ovarian follicles (Fig. 1B). However, the effect of IGF-I on steroidogenesis requires further investigation because, in all incubations where we attempted to evaluate it, concentrations of 20β-S and 17,20β-P in the culture medium were below detection limits of the respective RIAs. Changes in 17,20β-P concentrations could be important because this steroid is a potent stimulator of GVBD in vitro in *Morone* species. Because, unlike the case for 20β-S, receptors for 17,20β-P could not be detected on striped bass ovarian membranes, we believe that 17,20β-P is a biosynthetic precursor to the *Morone* MIH, 20β-S [28–29]. The two progestins were detectable in culture medium of tissues incubated with hCG in the present experiments on white bass, indicating that

the tissues were capable of producing the hormones but that IGF-I is not a potent regulator of MIH production in this species. The effects of IGF-I on MIH production seem to vary among fish species. For example, IGF-I has been shown to increase MIH production by striped bass follicles [15] and to potentiate GtH-induced MIH production by coho salmon (*Oncorhynchus kisutch*) follicles [21, 22], but IGF-I does not appear to influence follicular steroidogenesis in the mummichog (*Fundulus heteroclitus*) [33, 34].

Although 25 nM rhIGF-I in combination with 72.5 nM 20β-S induced oocyte GVBD in white bass follicles, a combination of the steroid plus 100 nM bovine insulin, or 1000 nM bovine insulin alone, failed to induce GVBD in the same experiment (Fig. 2). A combination of 1000 nM bovine insulin plus 290 nM 20β-S did induce oocyte GVBD, but less effectively than 100 nM rhIGF-I plus 290 nM 20β-S. The greater potency of IGF-I for inducing oocyte GVBD in the presence of the MIH suggests that IGF-I is acting in this regard via the IGF-I receptor and not through the insulin receptor. IGF-I and IGF-II have previously been shown to be more potent than insulin for inducing ovarian follicle maturation in fishes, including the striped bass [11, 15]. Furthermore, in several species of fish, IGF-I-receptor numbers and affinity were found to be greater than those of insulin receptors in preovulatory follicles [7, 8].

Increases in heterologous and homologous gap-junction coupling and increases in membrane MIH-receptor activity have been associated with GtH-induced acquisition of OMC in fishes, including the striped bass [15, 17, 20, 29, 35]. Furthermore, gap-junction coupling during follicular acquisition of OMC has been associated with the intensity of steroidogenesis and possibly of MIH production [1, 14, 15]. IGF-I also appears to be capable of inducing ovarian membrane MIH receptor activity in the spotted seatrout (*Cynoscion nebulosus*) [35] and of regulating coupling of heterologous and homologous gap junctions associated with OMC in ovarian follicles of red seabream [14].

In vitro treatment with *n*-alkanols (1-heptanol and 1-octanol), which function as gap-junction uncouplers, inhibited oocyte GVBD induced by all of the hormones and hormone combinations that were able to induce GVBD in white bass. The *n*-alkanols also inhibited hCG-induced follicular 20β-S and 17,20β-P production (Figs. 3 and 4). Similar effects of these *n*-alkanols on GtH-induced oocyte GVBD and on follicular steroid production were observed in the striped bass [15]. These observations are consistent with GtH and IGF-I inducing gap-junction coupling as part of the process of acquisition of OMC by white bass follicles, as has been shown for the red seabream [14]. However, the *n*-alkanols did not alter IGF-I-induced oocyte GVBD in the closely related striped bass [15], which supports our contention that differences in the mechanisms of IGF-I action in follicle maturation have rapidly evolved in the genus *Morone*. However, some ability of IGF-I to increase gap junction contacts in striped bass follicles cannot be completely ruled out at this time. It may be possible that IGF-I has this action to some extent in both species and that the apparent difference between white bass and striped bass results from differences between species in regulation of MIH activity by IGF-I or GtH. The effects of IGF-I on membrane MIH-receptor density or ligand-binding characteristics were not addressed in the present study.

The induction of follicle and oocyte maturation by GtH in lower vertebrates generally requires both de novo gene transcription and mRNA translation [1, 2, 3]. Evidence suggests that induction of OMC and of MIH production by GtH also requires de novo transcription and translation [4, 17, 36, 37]. However, MIH-induced resumption of oocyte meiosis (GVBD) requires only translation for MPF activation, specifically, for expression of cyclin B [4]. Actinomycin D and cycloheximide have been shown to inhibit hCG-induced oocyte GVBD and follicular 20β-S and 17,20β-P production in striped bass ovarian fragments in vitro [15]. Cycloheximide has been shown to have similar effects on white bass ovarian fragments, but the effects of actinomycin D were not examined in this species before the present study [28].

In white bass ovarian fragments, hCG-, 20β-S- and rhIGF-I plus 20β-S-induced GVBD were inhibited by cycloheximide, which is consistent with inhibition by cycloheximide of MIH production in response to GtH and of mRNA translation required for MPF activation by MIH. An inhibition of OMC by cycloheximide cannot be inferred from these results because induction of OMC requires MIH action to be observed. Actinomycin D added to the culture medium inhibited hCG- and hCG plus 20β-S-induced GVBD but not rhIGF-I plus 20β-S-induced GVBD. These data suggest that de novo transcription is required for GtH- but not IGF-I-induced acquisition of OMC in the white bass. Similar findings in the red seabream [12] led Patiño and Kagawa [14] to postulate that a transcription-dependent event in the induction of OMC by GtH is follicular production of IGF-I. The effects of GtH on ovarian IGF-I production remain to be investigated in *Morone* species and in red seabream.

The particularly strong response to IGF-I of white bass ovarian tissues, combined with the ability of IGF-I to induce OMC without gene transcription, is compatible with the idea that de novo transcription of mRNA for MIH receptors or for connexin proteins responsible for coupling of gap junctions is not required for the induction of OMC in white bass. Alternatively, before the tissues being removed for culture, GtH may have already induced some of these changes associated with OMC. For example, GtH may have induced transcription of MIH-receptor mRNA but not connexin mRNA, or vice versa, before the tissues were removed for incubation.

Two major signaling pathways for IGF-I receptor-mediated actions, the PI 3-K and MAPK pathways, are active in meiotic maturation of oocytes [38–40]. The MAPK cascade may reside downstream of PI 3-K activation [40–43]. The MAPK pathway was shown to be unnecessary for or unable to transduce signals capable of inducing oocyte maturation in goldfish [44]. Therefore, we investigated the effects of PI 3-K inhibitors on hormonal induction of follicle maturation in *Morone* species. We previously demonstrated that the PI 3-K inhibitors, wortmannin and LY 294002, inhibit in vitro rhIGF-I-, hCG-, and 20β-S-induced oocyte GVBD as well as follicular 17,20β-P production in striped bass ovarian fragments [16]. In white bass in the present study, wortmannin and LY 294002 dose-dependently inhibited hCG-, hCG plus 20β-S-, and IGF-I plus 20β-S-induced GVBD in MIH-incompetent ovarian fragments (Figs. 6 and 8). Wortmannin and LY 294002 also inhibited hCG- and, less consistently, 20β-S-induced GVBD in MIH-competent tissues (Figs. 7A and 9). In most of these experiments, concentrations of 20β-S in the culture medium were below detection limits of the respective RIA, but we did observe attenuation of hCG-induced production of 17,20β-P by tissues incubated with 50 μM LY 294002, for which the GVBD response is shown in Figure 9B (steroid data not shown). We did not, however, observe any effect of 100

nM wortmannin on 20β-S levels measured in the culture medium of tissues incubated with hCG in another experiment, results of which are shown in Figure 7B. In this experiment, there was a significant attenuation of follicular 17,20β-P production by wortmannin. Although inhibitory effects of PI 3-K inhibitors on MIH or MIH precursor production are possible in the white bass, our observation that wortmannin inhibited hCG-induced oocyte GVBD even when follicular 20β-S production was not altered (Fig. 7, A and B) or when 20β-S was added to the incubation (Fig. 6) supports the concept that PI 3-K activity is required for actions of GtH on follicle maturation distinct from its induction of MIH production.

The effects of inhibiting PI 3-K activity on IGF-I plus MIH-, GtH-, GtH plus MIH-, and MIH-induced GVBD is consistent with the PI 3-K pathway being involved with inducing or supporting maintenance of some component of OMC. This component may involve induction or maintenance of gap-junction contacts in the follicle, as we have previously suggested for the striped bass [16]. It also is possible that PI 3-K may be involved with activation of MIH receptors. The importance of the PI 3-K pathway in MIH signal transduction has been controversial, with conflicting findings as to whether or not PI 3-K activity is required for MIH action [42, 43, 45–47]. Recent evidence supports the idea that, in *Xenopus*, membrane progesterone receptors transduce the MIH signal and interact with the PI 3-K pathway. Furthermore, inhibition of this pathway by wortmannin or LY 294002 results in a delay of progesterone-induced oocyte maturation and a blockage of insulin-induced oocyte maturation [43, 48]. It is thought that the maturation-inducing effects of insulin are transduced via IGF-I receptors, which are in greater abundance on the *Xenopus* oocyte than are insulin receptors [49]. The attenuating effects of the PI 3-K inhibitors were only observed when lower, more physiological concentrations of progesterone were used to induce oocyte GVBD [48], perhaps explaining why results from studies investigating the role of the PI 3-K pathway in MIH action in *Xenopus* have been somewhat inconsistent.

Unlike the case in *Xenopus* and in the congeneric striped bass, IGF-I could not induce oocyte GVBD in the white bass. These results suggest that, in white bass, activation of the PI 3-K pathway is not sufficient to induce the resumption of meiosis. In all of these species, PI 3-K activity may increase or sustain OMC to varying degrees. The clear contrast in IGF-I actions between the striped bass and the white bass suggests that comparing PI 3-K-associated components of follicle maturation in these two species will lead to a better understanding of the participation of PI 3-K in the acquisition of OMC and in the resumption of oocyte meiosis.

In summary, IGF-I can induce OMC in white bass ovarian follicles, but it cannot induce the resumption of meiosis by white bass oocytes. These actions of IGF-I in white bass contrast sharply with those found in the congeneric striped bass, in which IGF-I can induce the resumption of meiosis via steroid (including MIH)-independent mechanisms but is not able to induce complete OMC. In *Morone*, IGF-I appears to act through IGF-I receptors, is dependent on PI 3-K activity, and requires de novo translation but not transcription. Attenuation by PI 3-K inhibitors of the induction of OMC by GtH and the induction of meiotic maturation by MIH supports the idea that IGF-I may primarily operate downstream of these hormones and also may potentiate their actions in follicle maturation. The apparently extreme disparate actions of IGF-I between two closely related *Morone* species with very different reproductive life-history strategies is consistent with the notion that variation in the IGF system and its actions can contribute to differences in modes of ovarian follicle maturation among species.

Finally, it should be noted that the disparate actions of IGF-I among the temperate basses and other vertebrate species indicate that ways of exploiting endocrine systems regulating follicle maturation will differ among species in agriculture. As an example, we have shown that the early in vitro maturational response of striped bass ovarian follicles to IGF-I (GVBD) can be used as a spawning competency test to identify when striped bass broodstock become able to respond to the gonadotropin-releasing hormone analogue implants used to induce final maturation and spawning [23]. This test will obviously not work for white bass because IGF-I does not induce GVBD in vitro. However, because IGF-I can induce OMC but not resumption of meiosis in white bass, it may be possible to manipulate IGF-I levels in this species to better synchronize ovarian follicles for subsequent response to exogenous GtH or MIH, thereby improving egg yields or quality. In conclusion, a better understanding of the diverse roles that the IGF system plays in follicle maturation will lead not only to a better understanding of ovarian follicle maturation but also to a better understanding of the evolution of reproductive strategies and to the development of tools to assist agriculture.

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